

# **Kinematics of mouthbrooding in *Oreochromis niloticus* (Cichlidae)**

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**Short title:** kinematics of mouthbrooding

## 1    **Abstract**

2    Many species out of several different families of fishes perform mouthbrooding: one of the  
3    sexes protects and ventilates the eggs inside the mouth cavity. This ventilation behaviour  
4    differs from gill ventilation outside the brooding period, as the normal, small-amplitude  
5    suction-pumping respiration cycles are then alternated with acts including near-simultaneous  
6    closed-mouth protrusions and high-amplitude depressions of the hyoid. The latter is called  
7    churning, referring to its hypothetical function in moving around and repositioning of the  
8    eggs by a presumed hydrodynamic effect of the vivid shifts in volume along the mouth  
9    cavity. We tested the hypothesis that churning causes the eggs located posterior in the mouth  
10    cavity to move anteriorly away from the gill entrance. This would prevent or clear  
11    accumulations of brood at the branchial basket, which would otherwise hinder breathing by  
12    the parent. Dual-view videos of female Nile tilapias (*Oreochromis niloticus*) during  
13    mouthbrooding showed that churning involves a posterior-to-anterior wave of expansion and  
14    compression of the head volume. Flow visualization with polyethylene microspheres  
15    revealed a significant inflow of water entering the gill slits at the zone above the pectoral fin  
16    base, followed by a predominantly ventral outflow passing the ventrolaterally flapping  
17    branchiostegal membranes. X-ray videos indicated that especially the brood located close to  
18    the gills is moved anteriorly during churning. These data suggest that, in addition to mixing  
19    of the brood to aid its oxygenation, an important function of the anterior flow through the  
20    gills and buccal cavity during churning is to prevent clogging of the eggs near the gills.

## 21   **Summary statement**

22   Mouthbrooding cichlids regularly use posterior-to-anterior waves of head expansion and  
23   significant inflow through the opercular slits to move the brood anteriorly away from the  
24   gills.

## 25   **Key words**

26   cichlids, tilapia, ventilation, opercula, jaw protrusion, churning, hydrodynamics,  
27   biomechanics

## 28 **Introduction**

29 Mouthbrooding is the behaviour of fishes to protect and ventilate the eggs in the  
30 buccal cavity until they have developed into free-swimming fry (Keenleyside, 1991). This  
31 form of parental care is found in at least nine families of Teleostei, including numerous  
32 species from the family of cichlids (Cichlidae) (Oppenheimer 1970). Evolutionary transitions  
33 from closely guarding of the eggs in nests or crevices to become mouthbrooders occurred at  
34 least ten times in the history of cichlids (Goodwin et al. 1998). During mouthbrooding, the  
35 buccal cavity of the parent is brought into a typical, enlarged posture to accommodate more  
36 eggs (Oppenheimer and Barlow, 1968; Goedel, 1974). In the Nile tilapia (*Oreochromis*  
37 *niloticus*), the model species of the current study, this posture includes a slightly protruded  
38 premaxilla, a depressed hyoid (Fig. 1A) and abducted suspensoria (Fig. 1B). X-ray pictures  
39 with a radio-opaque fluid filling the buccal cavity show how drastically the buccal volume  
40 increases by this postural change (Fig. 1C), allowing large females of this species to brood  
41 more than 1500 eggs (Valentin et al., 2015).

42 Ventilation behaviour of the mouthbrooding parent is crucial for the survival of the  
43 parent fish and the young (Oppenheimer and Barlow, 1968). Oxygen for the eggs and the  
44 newly-hatched young needs to be supplied by flows of fresh water generated by the cranial  
45 movements of the mouthbrooder. Not surprisingly, ventilation behaviour during  
46 mouthbrooding differs from the repertoire of cranial motions observed outside the  
47 mouthbrooding period. In cichlids, two main behaviours are displayed in bouts of varying  
48 lengths (see Supplementary Video 1), and of which the occurrence percentages depend on the  
49 time in the brooding period (Oppenheimer and Barlow, 1968):

50 (1) Respiration. This behaviour corresponds to the gill-ventilating, suction-pump  
51 movements also performed when the fish are not mouthbrooding. Since a large  
52 variation exists in the amplitude of the cranial movements during respiration, a  
53 distinction is sometimes made between “active” respiration (including large-  
54 amplitude motions of the jaws and opercula) and “passive” respiration (involving  
55 small-amplitude jaw-motions and small opening of the gill slits almost exclusively  
56 by movement of the branchiostegal membranes) (Oppenheimer and Barlow,  
57 1968). During active respiration, Oppenheimer and Barlow (1968) described that,  
58 when the mouth is opened, the incoming water causes the uppermost eggs to move  
59 backward to the rear of the buccal cavity, while the eggs on the left side move in a

clockwise manner (anti-clockwise for the eggs on the right side) and return forward along the side of the mouth (Oppenheimer and Barlow, 1968).

(2) Churning. Churning involves a closed-mouth protrusion of the premaxilla, depression of the hyoid, and abduction of the opercula (Oppenheimer and Barlow, 1968). The name of this behaviour refers to its hypothetical function in moving the brood around in the buccal cavity. At the instant during churning when the mouth is open, the eggs have been observed to roll about in the mouth (Bearends and Baerends-van Roon, 1950). Abraham (1901) peered through the semi-transparent, extended skin beneath the lower jaw of *Pseudocrenilabrus philander*, and saw the hatched “wrigglers” being rushed to the front of the buccal cavity, after which they retreated back out of sight to the back (Abraham, 1901).

The above observations suggest that the main difference in terms of brood movement between active respiration and churning is an abrupt forward impulse that is given to the intra-oral water during churning. This forward displacement of the brood may play a role in preventing the eggs from clogging near the gills and thereby causing respiratory obstruction (Abraham, 1901). If so, churning has a comparable role as a “forward cough” (*sensu* Kuiper, 1907). Coughing is regarded as a normal part of the respiratory activity of most fish (Hughes and Adeney, 1977). Alternatively, if the mouthbrooder’s respiration flows would not be critically hindered by the eggs, the exclusive function of churning could be to mix the eggs to reposition those that were deprived from sufficiently oxygenating flows. It is also possible that churning has a dual function in providing both mixing of the eggs and preventing them from clogging near the gills.

However, the kinematics of the head parts, water, and brood has thus far not been described quantitatively. This limits our understanding of the functions of the alternation of respiration and churning during brooding, and how these functions are realised by the cranial musculoskeletal system. As the flow of water inside the mouth cavity is determined by the kinematics of the elements that influence the shape and size of the mouth cavity, as well as by the opening and closing of the potential inlets and outlets (i.e. mouth aperture and gill slits), we will take the first step towards a better understanding of these functional differences by comparing the cranial kinematics of respiration and churning in this study. Next, certain aspects of the resulting flows of water during churning will be more directly evaluated by flow visualisation experiments.

In this study, we aim to answer the following three questions: (1) given the hypothesised forward intra-oral flow, is a posterior-to-anterior wave of buccopharyngeal expansion present during churning, as opposed to a normal anterior-to-posterior wave during respiration? (2) Does churning move the eggs to the front of the mouth, and is this a movement that allows a rather homogenous scrambling of the eggs versus a more localised movement, focusing on the displacement of the eggs that are positioned near the branchial sieve? (3) As opening of the gill slits by opercular abduction is clearly present during churning (Oppenheimer and Barlow, 1968), does this imply an inflow of water through the gills slits? Such a flow could be useful to clear or prevent obstructions in the posterior buccal cavity resulting from egg accumulations.

## **Materials and Methods**

### Animals and experimental conditions

Three adult females ( $149 \pm 33$  g; mean  $\pm$  sd) and three adult males ( $190 \pm 29$  g) of *Oreochromis niloticus* (Linnaeus 1758) were kept as pairs in separate 120 l aquaria at 27°C, under a 12h-12h day-night cycle, and fed *ad libitum* with cichlid pellets. Males and females were separated by a grid to avoid bite wounds. When the female appeared ready to mate, the grid was removed and male and female were united until spawning completed. Next, the mouthbrooding female was transferred to a 35 l aquarium for the video-recording sessions to quantify mouthbrooding kinematics. During these sessions, the animals were gently constrained into a corner of the aquarium using grids so that the head was in the field of view of the cameras. One female of 136 g was used in the additional fluid visualisations and x-ray videos, for which it was transferred to a small aquarium with thin, radio-translucent, plexiglass walls for the duration of the recording.

### Analysis of cranial kinematics

Mouthbrooding was filmed from a lateral and ventral view with two JVC Everio GZ-GX1 cameras (JVCKENWOOD Corporation, Kanagawa, Japan) at 50 frames per second (1920 x 1080 pixels, shutter time = 0.01 s) for 20 minutes on day 1, 3, 5, 7, and 9 of the brooding period. Synchronisation ( $\pm 0.02$  s) between the frames from the two cameras was inferred from a LED-flash at the start of each recording. The grid used for position-constraining that was just behind the fish on the lateral-view images was used for scaling. From the 20 minutes of video of each of these five days, a single cycle of churning and

ventilation was selected in which (1) the head was positioned central in the view of both cameras, and (2) the head showed negligible roll, yaw or pitch.

The two-dimensional coordinates of eleven anatomical landmarks (shown in the left column of Fig. 2) were determined on each video frame using Didge 2.3 (Alistair Cullum, Creighton University, USA). From these coordinates, six kinematical variables were determined as distances between two of these landmarks minus the minimum distance of the entire cycle: (1) mouth opening, (2) premaxilla protrusion, (3) hyoid depression, (4) suspensorium abduction, (5) branchiostegal membrane abduction, and (6) operculum abduction. The precise meaning of these variables is illustrated graphically in Fig.2 (left column). Digitization noise was filtered on the distance versus time profiles using a zero-phase shift, fourth-order low-pass Butterworth filter with a cut-off frequency of 3 Hz.

In order to compare the kinematics of respiration with that of churning in a standardised way, each video frame was assigned a “relative time”. The relative time duration of the full motion cycle is 100%, and the start of mouth opening is set as relative time = 0%. This allows calculation of the average kinematic profiles for each individual (N = 5, 4 and 4 for each behaviour; no data for day 5 in two of the three individuals) without generating potentially confusing time-averaged profiles with multiple peaks due to differences in duration of the cycles in absolute time. To test whether cycle duration and the amplitude of the quantified motions differed between the two behaviours (respiration and churning), a two-way ANOVA was used with individual as the second factor. Two variables, maximum opercular abduction and maximum protrusion, were log-transformed to pass the normality criterion (Shapiro-Wilk  $P > 0.05$ ). Equality of the variances ( $P > 0.05$ ) was always met. The interaction effect of factors individual and behaviour was included in the model but was never significant. Statistics were performed using SigmaPlot 11.0 (Systat Software Inc., Germany).

#### External flow visualisation

To visualise the inflow and outflow through the opercular slits, bright yellow polyethylene microspheres with a density of  $1.00 \text{ g cm}^{-3}$  and a diameter of 425-500  $\mu\text{m}$  (Cospheric LLC, Santa Barbara, USA) were added to the water of the small filming aquarium. Close-ups of the opercular region were filmed at 250 frames per second using a Redlake M3 camera. Two LED-panels (Falcon Eyes, Hong-Kong) provided the necessary illumination. To describe the general pattern of flow outside of the opercular slits during

churning, the best video (i.e. with good image sharpness and a high number of particles in the region of interest) was selected on which forty-nine individual particles were tracked.

### X-ray video analysis

We managed to place a small, wooden sphere with a fragment of steal in the centre into the buccal cavity of a mouthbrooding female and record its motion during nine sequences of churning using high-speed X-ray video. This experiment was the only successful one of five attempts. In the unsuccessful experiments, the brooding females responded to either sedation (using MS222) or the manipulation (forcing the dummy egg held by forceps into the mouth) by expelling all the eggs. The sphere is larger than the eggs (diameter = 4.5 mm vs. approximately 2.6 mm for an egg), and has a higher density despite the positively buoyant wooden edges. However, inducing accelerated water movement in a cup containing the sphere together with a large number of eggs after the experiment showed that the sphere only minimally lagged behind the motion of the eggs, and kept its original position in the centre of the pack of egg even after several trials. Consequently, the path of the sphere should be a good approximation of the path of the eggs in its vicinity.

Lateral-view X-ray videos were filmed at 500 frames per second with a Redlake MotionPro camera (1280×1024 pixels; Redlake, San Diego, CA, USA) attached to the image intensifier of a Philips Optimus M200 X-ray system (Royal Philips Electronics, Eindhoven, The Netherlands). The path of the sphere was determined by frame-by-frame, manual digitisation of the position of the sphere using Didge 2.3. The coordinates of two additional landmarks were determined on each video frame to re-calculate the path of the sphere in a head-bound frame of reference: a landmark in the centre of the otolith (reference frame origin), and one at the anterior tip of the vomer (defining the X-axis). The Y-axis was perpendicular to the X-axis and pointed dorsally.

## **Results**

### Cranial kinematics

The average kinematic profiles of the three individuals showed a consistent pattern within each behavior (i.e. churning and respiration) when time was scaled to cycle duration and a relative time ( $t_{rel}$ ) of 0% was set at the start of mouth opening (Fig. 2). A churning cycle lasted on average  $1.0 \pm 0.3$  s (mean  $\pm$  s.d.), a respiration cycle  $0.9 \pm 0.4$  s. Although cycle duration significantly differed between the individuals ( $F_{2,1} = 4.36$ ,  $P = 0.0027$ ), it did

not differ significantly between churning and respiration ( $F_{1,2} = 0.99$ ,  $P = 0.33$ ). A narrow opening of the mouth was observed during churning ( $1.4 \pm 0.3$  mm) as well as during respiration ( $1.0 \pm 0.2$  mm), which did not differ significantly between churning and respiration ( $F_{1,2} = 1.58$ ,  $P = 0.23$ ). However, peak mouth opening is reached about halfway the cycle duration during churning ( $t_{rel} = 48 \pm 3\%$ ), but later during respiration ( $t_{rel} = 62 \pm 8\%$ ) (Fig. 2A,G). Upper jaw protrusion was virtually absent during respiration ( $0.8 \pm 0.5$  mm) (Fig. 2H), but as churning was *a priori* identified when considerable protrusion occurred ( $2.0 \pm 0.9$  mm), the latter was obviously significantly higher ( $F_{1,2} = 32.0$ ,  $P < 0.001$ ) (Fig. 2B).

Churning involved larger expansions of the buccal and opercular cavities compared to respiration. A consistent pattern of depression of the floor of the buccal cavity at the level of the hyoid tip was only observed during churning ( $2.0 \pm 0.7$  mm) (Fig. 2C), not during respiration (Fig. 2I). Peak values of hyoid depression were thus significantly higher during churning ( $F_{1,2} = 8.95$ ,  $P = 0.007$ ). Also abduction of opercula ( $F_{1,2} = 6.78$ ,  $P = 0.017$ ; Fig. 2E,K) was significantly larger during churning ( $2.8 \pm 1.2$  mm) compared to respiration ( $1.7 \pm 1.1$  mm). Abduction of the suspensoria followed the same trend (churning:  $1.7 \pm 0.7$  mm, respiration;  $1.1 \pm 0.7$  mm), but this difference was not great enough to exclude the possibility that it is just due to random sampling variability after allowing for the effects of differences in individuals ( $F_{1,2} = 3.98$ ,  $P = 0.060$ ).

As hypothesized, a posterior to anterior wave of expansion was indeed present during churning, but not during respiration. During churning, first the opercula reach their peak abduction ( $t_{rel} = 63 \pm 10\%$ ; Fig. 2F), followed by the peak abduction of the suspensoria ( $t_{rel} = 77 \pm 6\%$ ; Fig. 2D), then peak hyoid depression ( $t_{rel} = 90 \pm 5\%$ ; Fig. 2C), and finally peak upper jaw protrusion ( $t_{rel} = 93 \pm 8\%$ ; Fig. 2B). The mouth starts to close ( $t_{rel} = 48 \pm 3\%$ ) before the expansion wave starts at the posterior side of the head, and can safely be considered fully closed from a relative time of about 80% onward (Fig. 2A) when the expansion wave reaches the anterior side of the head. Lateral flapping of the ventral portion of the branchiostegal membrane was not part of this expansion wave, as it occurred later ( $t_{rel} = 98 \pm 3\%$ ) while the other elements were compressing the buccal and opercular cavity. During respiration, the instant of mouth opening ( $t_{rel} = 62 \pm 8\%$ ; Fig. 2G) on average shortly preceded the abduction of the suspensoria (Fig. 2I) and opercula (Fig. 2K) (both at  $t_{rel} = 72 \pm 3\%$ ), after which finally the abduction of the ventral part of the branchiostegal membrane occurred (peak at  $t_{rel} = 98 \pm 3\%$ ).



## Water flow at the gill slits

Position tracking of polyethylene microspheres using high-speed video showed the pattern of inflow and outflow at the opercular and branchiostegal slits illustrated in Figure 3 and Supplementary Video 2. When the operculum is abducted, the branchiostegal membrane connected to the vertical, posterior part of the operculum edge flaps inward towards the gills. During this phase, water is sucked towards and into the opercular cavity through the gill slit (Fig. 3A). This inward flow continued during the approximate first 0.1 s of the opercular adduction (Fig. 3B). No spheres were observed to enter at the ventral side of the gill slit below the base of the pectoral fin. Next, the branchiostegal membrane at the opercular edge flaps outward (i.e. posteriorly away from the gills) and the first microspheres are observed to exit the gill slit (Fig. 3C). Many more spheres exited later when the dorsoposterior part of the branchiostegal membrane slowly moved inward again. Relatively few spheres were observed exiting above the base of the pectoral fin (4 out of 18): most of these were released in a jet of rotating flow ventral of the head (Fig. 3D). The above pattern was confirmed in many other videos that were recorded.

## Egg movement during churning

During nine churning acts of one individual, the paths traveled by a sphere with a radio-opaque centre inserted into the buccal cavity were determined (Fig. 4). The most posterior-starting path from the nine observations (path “1” in Fig. 4B), showed a considerable anterior movement until shortly after the instant of peak jaw protrusion. Next, the sphere moved a short distance back towards the posterior side of the head, but the net displacement was 20% of head length in the anterior direction. A consecutive act of churning (path “2” in Fig. 4B) brought the sphere a similar distance forward, after which it moved ventrally towards the floor of the buccal cavity at the level of the hyoid (Supplementary Video 3 shows acts “1” and “2”). Following were a series of paths that moved with the depression and elevation of the hyoid (paths “2” to “9”; Fig. 4B) with the exception of one (path “6”; Fig. 4B) where the sphere was moved antero-dorsally up to the mouth. No movement of the sphere could be observed during the respiration cycles in between churning.

## **Discussion**

Our analysis confirmed each of the hypotheses put forward based on the qualitative descriptions in the literature (Abraham, 1901; Oppenheimer and Barlow, 1968). Our X-ray videos provided the first quantitative evidence for forward movement of small objects inside

the mouth during churning (Fig. 4). This confirmed the description by Abraham (Abraham, 1901) for *Pseudocrenilabrus philander* in our model species *Oreochromis niloticus*. As hypothesized to be necessary to cause such a flow, a posterior-to-anterior wave of buccopharyngeal expansion was indeed present during churning (Fig. 2). This expansion wave involved both abduction of the opercula, abduction of the suspensoria, depression of the mouth floor by the hyoid, and protrusion of the jaws. Also as hypothesised in the introduction, abduction of the opercula indeed resulted in a considerable inflow of water entering the opercular slits (Fig. 3).

Together, these results suggest that the role of churning is more than only a mixing of the eggs to reposition those that were deprived from sufficiently oxygenating flows. The anterior flow of water entering through the gills will continue to move forward along with the expansion wave towards the front of the buccal cavity during churning. We hypothesise that this flow is generated to unblock the path of respiratory flow into the gills by moving the brood that may gradually become tightly packed by the posterior flows during respiration, or to prevent such obstructions from forming. This was also proposed by Abraham (Abraham, 1901) writing that the fish is *relieving himself from the choking feeling he must have been constantly subjected to*. Because wriggling brood may less easily form a clogging near the gill entrance, this may also explain the decrease in the frequency of churning once the young hatch and become more mobile (Oppenheimer and Barlow, 1968). Since also slightly increased amplitude of respiration cycles can bring a large part of the eggs in constant spiralling motion (perhaps except the most posterior ones, but this cannot be observed), the need for churning only for this reason (as proposed by Oppenheimer and Barlow, 1968) seems unlikely. Such motion of the eggs during highly active respiration was observed looking into the temporarily opened mouth (Oppenheimer and Barlow, 1968), which was also observed for the Nile tilapia during this study.

An interesting finding is that the position along the gill slit of the inlet and outlet of water during churning is not the same. Flow enters into the opercular cavity at the dorsal part of the gill slit near the abducted opercula, but exits more ventrally where the branchiostegal membranes are connected to the hyoid (Fig. 3). This can be explained by the opercular region of the gill slit being opened actively (i.e. by force from the dilatator operculi and levator arcus palatini muscles; Anker, 1978) and closed actively (i.e. by force of the adductor opercula and the adductor arcus palatini muscles; Anker, 1978), while the ventral region acts purely as a passive valve. Suction will be created during the opercular abduction phase,

resulting in a lower water pressure inside compared to outside of the head. Such a pressure gradient will automatically close the ventral branchiostegal valve. When afterwards the head is compressing and pressure becomes higher inside than outside of the head, the water pressure will push the passive valve open but cannot exit more dorsally on the gill slit because the opercula are firmly adducted at this instant. As a result, the intra-oral flow during churning will not be mirrored during the phases of anterior *versus* posterior flow, which may be important to guarantee a net forward displacement of the brood.

It cannot be excluded that churning also contributes to respiration of the mouthbrooder. Since the posterior-to-anterior expansion wave continued up to the protruding mouth region (Fig. 2) and forward displacement of the eggs was observed (Fig. 4), it is likely that most of the water entering the opercular cavity will pass the gill lamellae and flow into the buccal cavity. Respiration through an opercular inflow of water has been observed in other fishes, generally in species that live at the bottom and have a ventral mouth. For example, sturgeons (*Acipenser transmontanus*) for which inflow through the mouth was experimentally eliminated were able to draw water into their branchial cavities through openings in the upper regions of the opercular slits (Burggren, 1978). Astroblepid catfishes have freed their suckermouth (used during climbing) from its inhalatory function by a duplication of the gill openings: only inflow through the incurrent gill openings were observed (De Crop et al., 2013). However, due to the relatively high impulse given to the water during churning in *Oreochromis niloticus* (higher amplitude of the expansion wave compared to respiration; Fig. 2), shunting of water around the gills is possible (Strother, 2013a; Strother, 2013b). Consequently, it is unsure whether the flows generated during churning are useful for the gills of the parent fish to extract oxygen from. Despite that the generation of such high-impulse flows is expected to result in a considerable energetic cost to the mouthbrooder, an oxygen consumption study of the cichlid *Pseudocrenilabrus multicolour* showed that the energetic cost of mouthbrooding is limited to an increase of only a few percentages (Mrowka and Schierwater, 1988).

The posterior-to-anterior wave of cranial expansion during churning will require a different motor program (i.e. sequence of muscle activation) compared to the more general anterior-to-posterior waves of expansion that is much more commonly observed, for example during respiration or suction-feeding. This raises the question on the origin of such a reversed activation sequence, namely whether this neuro-motor pattern is a novel trait that appeared at the origin of mouthbrooding. This seems unlikely as other actions that are more

common in fishes share notable similarities. As mentioned in the introduction, coughing is regarded as a normal part of the respiratory activity of most fish (Hughes and Adeney, 1977; Summers and Ferry-Graham, 2001), and often these are “forward coughs” (Kuiper, 1907). Oral transport of offspring between excavated pits by substrate guarders is assumed to have provided the first step towards the evolution of mouthbrooding (McConnell, 1959; Goodwin et al., 1998). Forward coughs may be used to release the offspring after transport. However, the mouth is not closed in such coughs. This is a significant difference from the closed-mouth protrusion during churning. During intra-oral manipulation of food, however, we do see closed-mouth protrusions that appear remarkably similar to churning. The kinematics of closed-mouth protrusion acts during food processing have been described in detail in cyprinid fish (Callan and Sanderson, 2003; Gidmark et al., 2012). Yet, it remains to be shown whether the cranial kinematics of these actions during intra-oral food manipulation are identical to churning. Nevertheless, it is likely that the ability to perform actions analogue to churning preceded the evolution of mouthbrooding.

The functional morphology of posterior-flow-driven transport of food inside the mouth cavity by aquatic fishes has been studied intensively during the past decades (Day et al., 2015). Consequently, the sequence of motion of the cranial elements involved during this typical transport of food transport towards the oesophagus entrance is well-known. In contrast, relatively few studies dealt with other types of intra-oral, hydrodynamic manipulations of items inside the mouth cavity (e.g., Liem, 1979; Drucker and Jensen, 1991; Konow and Sanford, 2008; Gidmark et al., 2012). How fish manage to separate food from debris, or position a bolus in between their pharyngeal jaws by hydrodynamic actions remains largely unknown (but see Drucker and Jensen, 1991). Such behaviors, together with the common capacity of fish to spit out undesirable items, and the churning kinematics described in the present study, illustrate the versatility of the buccal apparatus of fishes beyond generating the typical anterior-to-posterior flows during suction feeding. As argued above, this versatility might have paved the way for the evolution of mouthbrooding in cichlids.

In conclusion, our kinematical analysis revealed new insights on the role of churning during mouthbrooding, and how churning is performed by the cranial musculoskeletal system in the Nile tilapia. We described how this species alternates between different motion sequences of its cranial elements when switching between respiration and churning during mouthbrooding. During churning, a posterior-to-anterior wave of cranial expansion and then compression was used instead of the more common, anterior-to-posterior wave that is used by

fish for respiration and suction feeding. This reversed motion sequence reverses the direction of water flow: we observed an inflow of water through the opercular slits as well as a net anterior displacement of small objects initially located at the back of the mouth cavity. This anterior flow of water can help to avoid respiratory flow obstructions when too dense accumulations of eggs are formed near the gills. Whether also the mixing of the eggs due to churning in combination with the inflow of fresh water from the back of the head is important for the oxygen supply to the brood could be a topic for future research.

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### **Competing interest statement**

No competing interests declared.

### **Author contributions**

S.V.W, G.D.B., D.A. and P.A. designed the study. M.D. and H.J.L. made the dual-view videos. I.J. analysed the kinematics and performed the flow visualisation. S.V.W performed the particle tracking, recorded and analysed the X-ray videos, and wrote the manuscript; all authors discussed the results and commented on the manuscript.

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## Figure captions

Figure 1: Mouthbrooding posture of the head in the Nile tilapia. Mimicking our video images of this species during mouthbrooding, the head of a dead specimen was forced into this posture and subjected to laser scanning (A: lateral view, B: dorsal view) and X-ray imaging using a dense bariumsulfate solution filling the buccal cavity (C). Red lines show the outlines of the buccal cavity.

Figure 2: Cranial kinematics of churning (A-F) and respiration (G-L) in three individual Nile tilapias. Mean kinematic profiles ( $N = 4$  or  $5$ ) per individual (colour coded) with standard deviation ranges (shaded area bordered by dashed lines) are shown in function of a relative time (% of cycle duration) for the six variables illustrated on the left.

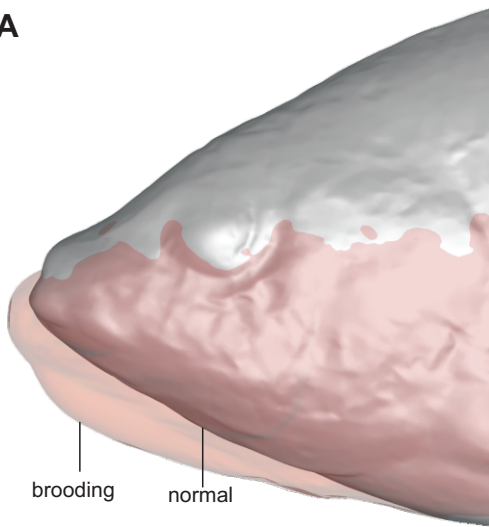
Figure 3: Paths of the tracked microspheres that flow in and out of the gill slits during a representative churning sequence. Inflowing paths are shown in red, outflowing paths in green. Paths with dotted lines are continued in the next image. The sequence is subdivided into four phases (A-D) depending on the kinematics of the operculum and the dorsoposterior part of the branchiostegal membrane, as shown by the ranges indicated by the horizontal arrows at the top. The end time of each interval is shown on the upper right of the images (start of the first interval is at 0 s).

Figure 4: Paths of a small sphere containing a radio-opaque marker during churning as determined based on nine high-speed x-ray videos. The head-bound frame of reference and the monitored region is shown in (A). In (B), path lines for each churning sequence are colored and numbered. The direction of movement and the position of the sphere at three instants are indicated by arrows (red arrow, 40 ms before the time of peak protrusion; black arrow, at the time of peak protrusion; green arrow, 40 ms after the time of peak protrusion).

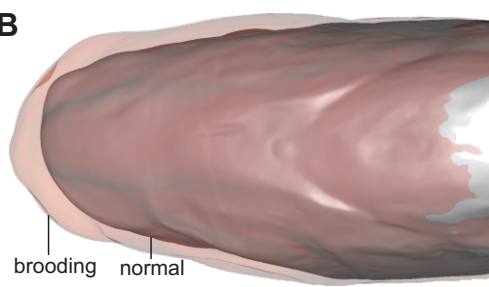


**FIGURE 1**

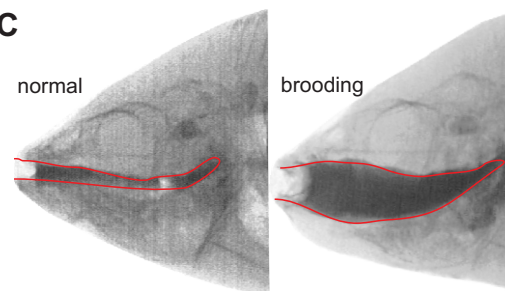
**A**



**B**



**C**



**FIGURE 2**

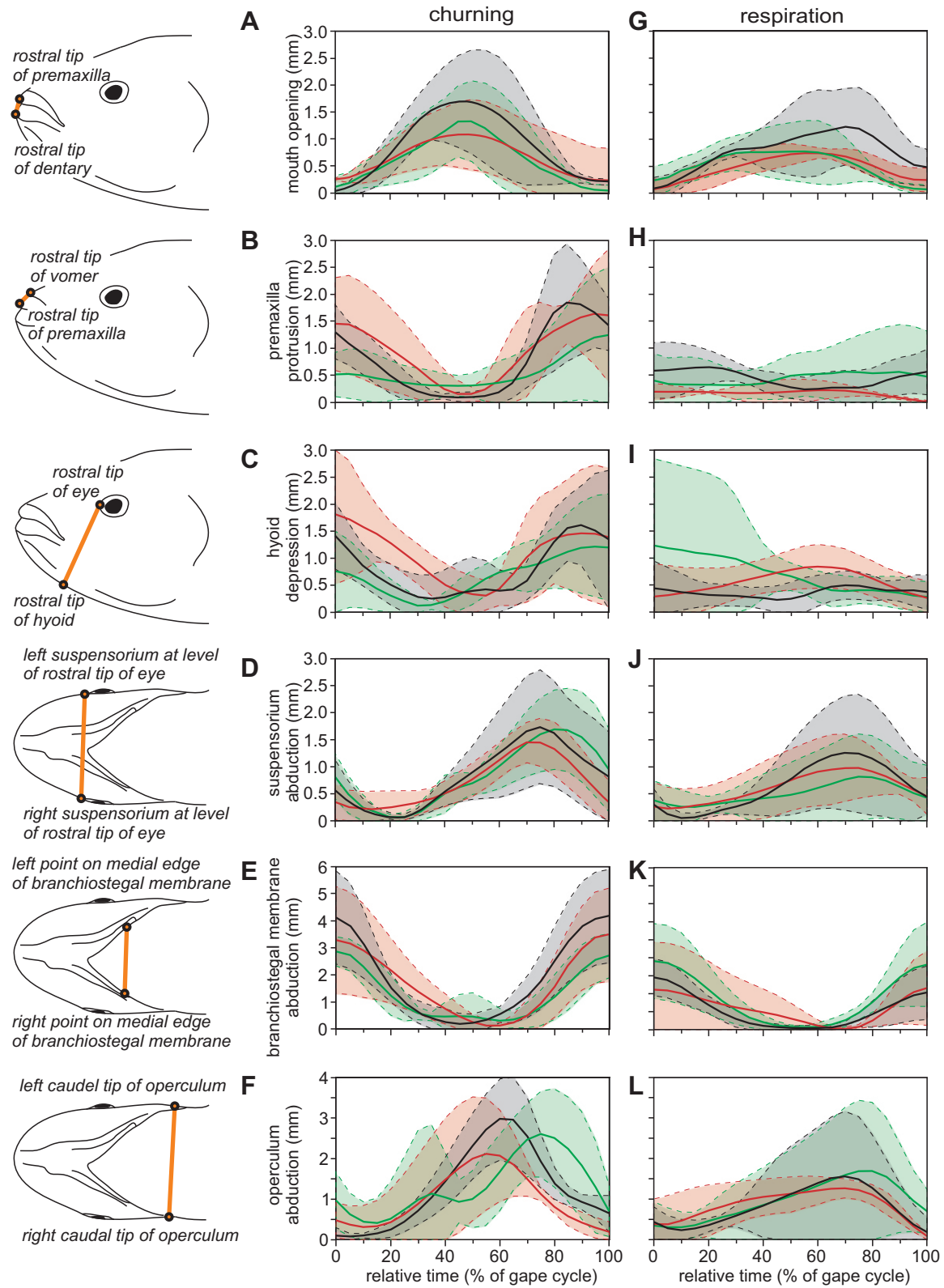


FIGURE 3

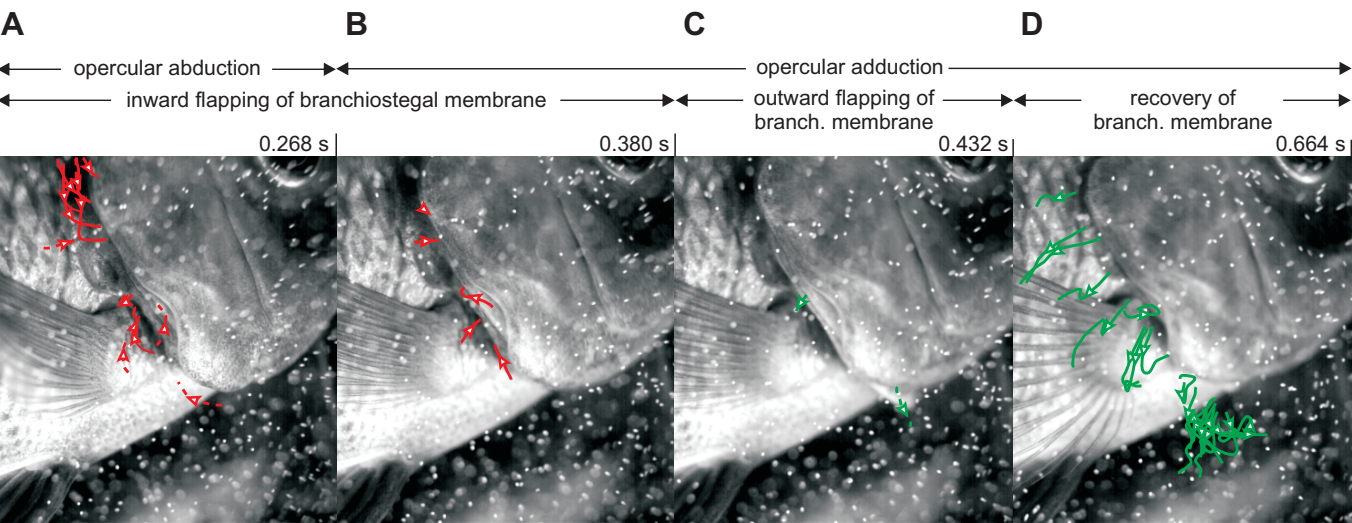


FIGURE 4

